High Expression of MMP-9 in Primary Tumors and High Preoperative MPO in Serum Predict Improved Prognosis in Colorectal Cancer with Operable Liver Metastases

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High expression of MMP-9 in primary tumors and high preoperative MPO in serum predict improved prognosis in colorectal cancer with operable liver metastases

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Short Title: Tissue MMP-9 and serum MPO predict prognosis in CRC with liver metastases

Keywords: Colorectal cancer; liver resection; MMP-9; MPO; prognosis

Abstract

Introduction: The liver metastases of colorectal cancer (CRC) can be surgically resected in selected cases with continuously improving results. Matrix metalloproteinases (MMPs) contribute to cancer invasion by degrading extracellular matrix, and elevated levels of MMP-2, MMP-8, and MMP-9 have been detected in several malignancies. Myeloperoxidase (MPO) is a mediator of tissue damage that can oxidatively activate latent MMPs. We evaluated the prognostic value of MMP-2, MMP-8, and MMP-9 in tissue samples of primary tumors and liver metastases and the pre- and postoperative serum levels of MMP-8, MMP-9, and MPO in CRC patients undergoing liver resection.

Methods: Tissue and serum samples were obtained from 111 patients, who had their primary colorectal tumors and liver metastases operated on at the Helsinki University Hospital between 1988 and 2007. Tissue expression of MMP-2, MMP-8, and MMP-9 in primary tumors and liver metastases was evaluated by immunohistochemistry. Pre- and postoperative serum concentrations of MMP-8, MMP-9, and MPO were determined using a time-resolved immunofluorometric assay (IFMA) or commercially available enzyme-linked immunosorbent assay (ELISA) kits. Clinical data were retrieved from patient records and the Central Statistical Office of Finland. Associations with disease-free survival (DFS) and overall survival (OS) were estimated using the Cox regression analysis and the Kaplan-Meier method.

Results: High expression of MMP-9 in colorectal tumor tissue associated with better DFS (P=0.010), and high preoperative MPO in serum with improved DFS and OS (P<0.001 and P=0.014, respectively). The prognostic significance varied according to gender, age, and the synchronicity of the liver metastases.

Conclusion: Low preoperative MPO in serum might identify the patients at a high risk of recurrence and death after resection of colorectal liver metastases. High expression of MMP-9 in colorectal tumor tissue and elevated preoperative MPO in serum indicate improved prognosis. The use of these biomarkers should be adjusted according to clinical characteristics.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer worldwide [1], and the factors affecting the course of the disease and prognosis are under continuous investigation. Treatment of stage IV disease has improved markedly in the last decades, but it still leads to death in almost 90% of cases [2]. Up to 75% of the patients with metastatic disease develop liver metastases [3], and the only curative treatment option is surgical resection. Only 10–20% of the liver metastases are operable at the time of diagnosis, but due to improved ongological treatment, up to 15% of the initially non-resectable metastases can be converted to resectable [4, 5]. However, more than half of the patients develop recurrence after liver resection [6].

The invasion of the primary tumor into the adjacent tissues is a critical step in cancer metastasis. Invasion requires remodeling and degradation of the extracellular matrix (ECM), in which proteolytic enzymes (cysteine-, serine-, aspartic-, and metalloproteinases) exert a significant role. Matrix metalloproteinases (MMPs) are a family of zinc-dependent genetically distinct but structurally related endopeptidases, which are able to degrade almost all extracellular matrix proteins, activate and process bioactive non-matrix biomolecules including growth factors and cytokines, and promote immune responses and angiogenesis [7]. MMP-2 and MMP-9 belong to the subgroup of gelatinases, and MMP-8 (collagenase-2) to that of collagenases. The activity of MMPs is controlled by tissue inhibitors of metalloproteinases (TIMPs), especially TIMP-1 [8, 9]. In addition, tumor-associated trypsin inhibitor (TATI) can inhibit the action of trypsin-2, and thus, the activation of collagenolytic latent MMPs [10]. MMPs are upregulated in several different inflammatory processes as well as in cancer, and their role can be either proinflammatory or anti-inflammatory depending on the condition and its stage or phase [11]. It is known that tumor development triggers inflammation, but the role of MMPs in regulating the inflammatory processes is yet unestablished.

Overexpression of MMP-2 and MMP-9 in tumor tissue has been associated with poor prognosis in recurrent glioma [12]. MMP-2 has indicated poor prognosis also in colorectal cancer [13] and gastric cancer [14, 15]. The role of MMP-9 has been controversial, as high tumoral expression has been shown to indicate shortened survival in head and neck cancer [16], in gastric cancer in one study [17], and in primary colorectal cancer

[18-20], but it has also been associated with better survival in Dukes' B colorectal cancer [21] and in extrahepatic bile duct cancer [22].

MMP-8 expression in tumor tissue has either not been prognostic or it has been variable [23]. However, there is evidence that MMP-8 prevents metastasis through modulation of tumor cell adhesion and invasion [24]. It has been shown to protect against the development of skin tumors [25] as well as lymph node metastasis in breast cancer [24], and in squamous cell tongue cancer, high expression in cancer cells has been associated with improved survival [26].

Elevated levels of MMP-2, MMP-8, and MMP-9 in serum have been observed in several different cancers. Elevated serum concentrations of MMP-8 have associated with worse prognosis in colorectal cancer [27-29], hepatocellular cancer [30], and melanoma [31]. Interestingly, in the study by Laitinen et al. [32], both high and low serum levels of MMP-8 associated with worse survival in gastric cancer. It has been suggested that the serum levels of MMP-2 and MMP-9 might be used for identifying the patients at a high risk of recurrence in CRC [33], and MMP-9/TIMP-1 ratio has been prognostic in hepatocellular cancer [30], but otherwise their significance is yet unclear.

Myeloperoxidase (MPO) is a heme-containing peroxidase that is expressed in neutrophils and monocytes. It is overexpressed in inflammatory diseases, and it acts as a mediator of microbial defense and tissue damage [34]. MPO is thought to contribute to pathogenesis [35], but on the other hand, it is needed for the defensive immune responses [36]. It catalyzes the production of hypochlorous acid (HOCl), which leads to reactions that generate reactive oxygen intermediates such as chloramines and free radicals [37, 38]. These products can attack extracellular tissues and be pathogenic [39]. MPO also activates proMMP-8 and -9 [40], and it has been shown that it regulates the activity of matrix MMP-7 *in vitro* [41]. On the other hand, it can oxidatively inactivate pathogenic microbes and TIMP-1 [42].

High tumor infiltration of MPO-expressing cells has been associated with significantly improved prognosis in colorectal cancer and breast cancer [43, 44]. The prognostic significance of serum MPO in cancer remains to be investigated, even though high serum concentrations have been associated with negative lymphovascular invasion in breast cancer [45].

In this study we evaluated whether the tissue expression of MMP-2, MMP-8, and MMP-9 in both primary colorectal tumors and liver metastases or the serum concentrations of MMP-8, MMP-9, and MPO associate with prognosis in CRC patients undergoing resection for liver metastases. Their prognostic significance was studied in the whole patient cohort and in specified subgroups. The aim was to assess whether these biomarkers are prognostic of possible recurrence or survival after liver resection.

Materials and Methods

Patients

This study included 111 patients that had both their primary colorectal tumors and liver metastases operated on at the Helsinki University Hospital area between the years 1988 and 2007. The primary colorectal tumors were operated between 1988 and 2007, and the liver metastases between 1997 and 2007. Tissue samples of both primary tumors and liver metastases were obtainable from 60 patients, samples of primary tumor only from 21 patients, and samples from liver metastases only from 30 patients. The serum samples were available from all patients (**Figure 1**). The liver metastases were considered synchronous, if they were diagnosed at the same time or within 6 months after the operation on the primary tumor, and as metachronous, if they were diagnosed later. Clinical data were retrieved from patient records, and information about the dates of death was obtained from the Central Statistical Office of Finland (TK-53-1004-9).

The study was approved by the Ethics Committee of the Helsinki University Hospital (IRB99/07/01, HUS531/E6/01, HUS460/E6/05, HUS323/13/3/2008, HUS242/13/03/02/2011, and HUS 226/E6/06, extension TMK02 §66 17.4.2013). Collection and analysis of tissue and blood samples were approved by the National Supervisory Authority for Welfare and Health (STM Dno 4858/04/047/08 and Valvira Dnro 10041/06.01.03.01/2012).

Preparation of tissue microarray samples

Formalin-fixed and paraffin-embedded tissue samples were obtained from the Department of Pathology of the Helsinki University Hospital. Representative tumor areas and healthy tissue areas were marked on immunohistochemically stained slides with the help of an experienced pathologist (JH). Six punches (1000 µm in diameter) were taken from each sample's tumor area, from the invasion front area when possible. The punches were taken with a semiautomatic tissue microarray instrument (TMA) (Beecher Instruments, Silver Spring, MD) and mounted on TMA paraffin blocks. Two series of blocks were constructed, each containing 3 tumor samples from every patient's 1) colorectal tumor tissue, 2) liver metastasis tissue.

Immunohistochemistry of MMP-2, MMP-8, and MMP-9

TMA blocks were cut into 4-µm-thick sections for immunohistochemistry. Sections were deparaffinized in xylene and then rehydrated through a graded alcohol series. For antigen retrieval, the samples were heated in Tris-ethylenediaminetetraacetic acid (EDTA) (pH 9) (MMP-2 and MMP-8) or in Tris–HCl (pH 8.5) buffer (MMP-9) for 20 minutes at 98°C in the PreTreatment module (Agilent Dako, Lab Vision Corp., Fremont, CA, USA). After inactivation of endogenous peroxidases, the sections were first incubated with a mouse monoclonal anti-MMP-2 antibody (Clone CA-4001; Lab Vision Corp., Fremont, CA, USA) diluted at 1:50 overnight, with a polyclonal MMP-8 antibody [46] diluted at 1:400 overnight, or with a mouse monoclonal anti-MMP-9 antibody (MS-817-PO; NeoMarkers, Fremont, CA, USA) diluted at 1:1500 for one hour using the Dako REAL Antibody Diluent S2022 (Dako), and then with the secondary antibody (Dako Real ENV rabbit/mouse HRP antibody) for 30 minutes. The sections were subsequently visualized with Dako Real ENV Dab chromogen kept on glass for 10 minutes and counterstained with Dako Mayer's Hematoxylin S3309. They were dehydrated through a graded series of water, ethanol, and xylene, and finally mounted using Pertex® Histolab mounting medium. The staining process was performed with the Lab Vision Autostainer 480 (LabVision Corp., Fremont, CA, USA).

Scoring

The immunohistochemically stained tumor samples were scored by two independent researchers (RP and JH) without knowledge of the patients' clinical outcome. In case of different scoring results, the samples were re-evaluated and a consensus score determined. Spots without cancer cells or with too few cells for adequate estimating were excluded.

The scoring of MMP-2 was based on the intensity of cytoplasmic staining in tumor cells as follows: 0: no positivity in any cancer cells; 1: mild staining in all or some cancer cells; 2: moderate staining in most cancer cells; and 3: strong staining in all cancer cells (**Figure 2**).

The scoring of MMP-8 was based on the amount of stained inflammatory cells (granulocytes) in immediate proximity to the tumor cells, and the scoring was defined as follows: 0: no staining or only one stained granulocyte; 1: some stained granulocytes; 2: several stained granulocytes; and 3: abundantly of stained granulocytes (**Figure 2**).

The scoring of MMP-9 was based on the intensity of granular cytoplasmic staining in the tumor cells as follows: 0: no staining; 1: scattered granules in some cancer cells; 2: granules in all or some cancer cells; and 3: several granules in all cancer cells (**Figure 2**). In liver tissue, no staining or only some granulary staining was seen as well as some stained inflammatory cells.

Serum samples

The serum samples were taken in conjunction with the liver resection, before and approximately 3 months after the resection. The median time between preoperative sampling and the liver resection was 14 days (interquartile range [IQR] 8–22 days), and the median time between the resection and postoperative sampling 92 days (IQR 88–97 days). The samples were stored in -80˚ Celsius until analyzed.

The serum concentrations of MMP-8 were measured by a time-resolved immunofluorometric assay (IFMA) as described previously [47]. The monoclonal MMP-8 specific antibodies 8708 and 8706 (Oy Medix Biochemica Ab, Espoo, Finland) were used as a catching and a tracer antibody, respectively. The tracer antibody was labeled using europium chelate [48]. The assay buffer contained 20 mM Tris-HCl (pH 7.5), 0.5 M NaCl, 5 mM $CaCl_2$, 50 µM $ZnCl_2$, 0.5% BSA, 0.05% sodium azide, and 20 mg/l diethylenetriaminepentaacetic acid (DTPA). Samples were diluted in assay buffer and incubated for 1 hour followed by incubation for 1 hour with the tracer antibody. Enhancement solution was added, and after 5 minutes, fluorescence was measured using a 1234 Delfia Research Fluorometer (Wallac, Turku, Finland). The specificity of the monoclonal antibodies against MMP-8 corresponded to that of polyclonal MMP-8. The interassay coefficient of variation (CV) % was 7.3% and the detection limit 0.08 ng/ml.

The serum concentrations of MMP-9 and MPO were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturers' instructions (MMP-9: Amersham

BioSciences UK Ltd., Buckinghamshire, UK; MPO: Immundiagnostik AG, Bensheim, Germany). The detection limits were 0.6 ng/ml for MMP-9 and 1.6 ng/ml for MPO [49, 50].

Statistical analyses

Statistical analyses were carried out and survival curves were created using SPSS Statistics 25 (SPSS Inc., Chicago, IL, USA). A *P*-value of less than 0.05 was considered significant, and statistical trend was defined as $P<0.1$. DFS and OS were calculated from the date of liver resection. DFS was defined as time to any recurrence of CRC or death of any cause, and OS as time to death of any cause. All patients were censored at the end of follow-up. The data cut-off date was January 11th, 2019.

The correlations between the immunohistochemical expression and serum concentrations were calculated with Spearman's rank correlation test and their associations with clinical parameters with Fisher's exact test. The biomarkers' distributions in tissue and serum in the specified subgroups of patients were studied using Mann-Whitney U test. Survival curves were created with the Kaplan-Meier method, and the differences between the groups were assessed using the log-rank test. Univariate survival analyses were performed with the Cox proportional hazards model. Serum concentrations of MMP-8, MMP-9, and MPO were included in the Cox regression analyses as $\log_2$-transformed continuous variables, as they were non-normally distributed. Receiver operating characteristic (ROC) curves in relation to 5-year DFS were used for estimating the optimal cut-off values for serum MMP-8, MMP-9, and MPO.

All the subgroups of patients considered to have clinical relevance and containing enough patients for statistical reliability were included in the survival analyses. Thus, the tissue expression, serum concentrations, and the prognostic value of the biomarkers were evaluated in the following subgroups: gender (male vs. female); age at the liver resection (≤65 vs. >65 years); location of the primary tumor (rectum vs. colon); and the type of liver metastases (synchronous vs. metachronous).

Results

Patient characteristics

The patient characteristics are shown in **Table 1**. Altogether 57.7% of the patients were male. The primary tumor was located in the rectum in 48 (43.2%) cases and in the colon in 63 (56.7%) cases. Synchronous liver metastases were diagnosed in 66 (59.5%) patients. During the follow-up time after liver resection, 80 (70.1%) patients experienced recurrence. Of the recurrences, 18 (22.5%) were diagnosed within 3 months after resection, 52 (65.0%) within 3 years, and 10 (12.5%) later. The minimum follow-up time after liver resection for all living patients was 11.5 years.

Biomarkers in tissue and serum samples

The scoring results as well as the medians and IQRs of the serum concentrations are shown in **Table 2**. High MMP-2 expression was observed in 71/81 (87.7%) of the primary tumor samples and in 40/88 (45.5%) of the liver metastasis samples, and the expression in primary tumors was higher in colon cancer compared to rectal cancer (P=0.014). Tissue expression of MMP-8 was high in 43/81 (53.1%) of the primary tumors and in 33/88 (37.5%) of the liver metastases, and in synchronous disease, the expression in both primary tumors and liver metastases was lower than in metachronous disease (P=0.029 and P=0.039, respectively).

The expression of MMP-9 was high in 30/81 (37.0%) of the primary tumors and in 20/88 (22.7%) of the liver metastases. The expression in primary tumors was lower in ≤65-year-old patients compared to older ones (P=0.050) and also in synchronous disease compared to metachronous disease (P=0.037).

It is worth noticing that in patients with low MMP-9 expression in primary tumors, the median time between the operation on the primary tumor and the liver resection was 7.9 months (IQR 4.1–14.2), while it was 15.1 months (IQR 5.4–20.3) in those with high expression, a difference that was statistically significant (P=0.047, Mann-Whitney U test). In patients with low MMP-8 expression in primary tumors, the median time between the operations was 7.6 months (IQR 3.6–14.5) compared to 12.5 months (IQR 6.2–18.2) in those with high expression (P=0.045, Mann-Whitney U test).

The serum concentrations of MMP-8, MMP-9, and MPO did not associate with any of the investigated clinical parameters, and there were no statistically significant differences in their distributions.

Correlations and associations

MMP-2 expression in primary colorectal tumors correlated only with that of MMP-8 in primary tumors (rho -0.297, *P*=0.007), and the expression in liver metastases did not show statistically significant correlations. The expression of MMP-8 in primary tumors did not correlate with the other biomarkers, but there was an association with the type of liver metastases (synchronous/metachronous) (*P*=0.043, Fisher's exact test). MMP-8 expression in liver metastases correlated with preoperative serum MMP-8 (rho 0.334, *P* =0.002) and serum MPO (rho 0.218, *P* =0.044), and also with postoperative serum MMP-8 (rho 0.236, *P* =0.027). Low MMP-8 expression in the liver metastases associated with lower serum concentrations of MMP-8 pre- and postoperatively (*P* =0.004 and *P* =0.005, respectively; Mann-Whitney U test).

MMP-9 expression in primary tumors correlated with postoperative serum MMP-8 (rho -0.430, *P* =0.002), when only patients with synchronous liver metastases were included, as low tissue expression associated with higher serum concentrations (*P*=0.002; Mann-Whitney U test). Associations with the location of the primary tumor and the type of liver metastases (synchronous/metachronous) were also noted (*P*=0.031 and P=0.017, respectively; Fisher's exact test). MMP-9 expression in liver metastases did not show statistically significant correlations.

Besides the above-mentioned correlations, the serum values of MMP-8, MMP-9, and MPO all correlated with each other pre- and postoperatively (rho 0.197–0.701, *P*<0.001–0.001), as the lower serum concentrations associated with lower ones and higher concentrations with higher ones (all: *P*<0.001; Mann-Whitney U test). In addition, pre- and postoperative serum MMP-8 associated with the location of the primary tumor (*P*=0.034 and *P*=0.066, respectively; Fisher's exact test), and preoperative serum MPO with age (≤ or >65 years at liver resection) nearly significantly (*P*=0.080; Fisher's exact test).

Survival analyses

The disease-free and overall survival of the patients in different clinical subgroups are presented in **Table 3**. The median disease-free survival time in the whole cohort was 1.4 years (IQR 0.6–9.4), and the median overall survival time 5.7 years (IQR 2.7–13.4). Disease-free survival was 29.7% (33/111) at 5 years after resection and 20.7% (23/111) at the end of follow-up. Overall survival was 59.5% (66/111) at 5 years, 36.9% (41/111) at 10 years, and 30.6% (34/111) at the end of follow-up.

When compared with the log-rank test, DFS and OS were significantly shorter among elderly patients than among younger ones, when the age was >65 years at the time of the operation on the primary tumor (DFS: *P*=0.036; OS: *P*=0.013) or at the liver resection (DFS: *P*=0.014; OS: *P*=0.041). Patients with T3–4 primary tumors had worse survival than those with T1–2 tumors (DFS: *P*=0.015; OS: *P*=0.006), as did also patients with primary N1–2 status compared to N0 status (OS: *P*=0.011). In addition, having more than two liver metastases indicated shorter DFS nearly significantly (*P*=0.053). Gender, the synchronicity of the liver metastases, or perioperative chemotherapy did not associate with survival statistically significantly.

MMP-2, MMP-8, and MMP-9 in tissue samples and survival

For the survival analyses, the expressions of MMP-2, MMP-8, and MMP-9 in the tumor samples were grouped as “low” (score 0–1) and “high” (score 2–3). The results of the univariate Cox regression analyses are shown in **Table 4**, and those attained using the Kaplan-Meier method are presented in the following section.

The expression of MMP-2 and MMP-8 in either primary colorectal tumors or liver metastases did not associate with shorter DFS or OS, when the whole patient cohort was examined. In women, low MMP-2 expression in liver metastases associated with shorter DFS (*P*=0.032) and showed tendency towards shorter OS (*P*=0.055), but otherwise MMP-2 was not prognostic in the subgroups. High expression of MMP-8 in liver metastases indicated worse survival only in colon cancer (OS: *P*=0.046).

High MMP-9 expression in primary colorectal tumors, on the other hand, associated with improved DFS (*P*=0.010) in the whole cohort and also with better OS nearly significantly (*P*=0.067) (**Figure 3**). In addition,

high expression of MMP-9 in primary tumors indicated improved prognosis more strongly in the specified subgroups as follows: in women (DFS: *P*=0.005 and OS: *P*=0.014); in colon cancer (DFS: *P*=0.011; OS: *P*=0.037); and in metachronous disease (DFS: *P*=0.005; OS: *P*=0.032) (**Table 5**). MMP-9 expression in liver metastases did not associate with survival.

MMP-8, MMP-9, and MPO in serum samples and survival

The results of the univariate Cox regression analyses including the serum concentrations as $\log_2$-transformed continuous variables are shown in **Table 4**. In the Kaplan-Meier log-rank analyses, we used the cut-off level of 218.6 ng/ml for MPO based on the ROC curve analysis, and that of 77.7 ng/ml for MMP-9, as it was the mean of the pre- and postoperative medians and close to the cut point provided by the ROC curves. For MMP-8, we decided to use the tertiles of the pre- and postoperative values as cut points, as no statistically significant cut-off level was found. Thus, the cut-off values were 29.6 and 76.2 ng/ml for preoperative MMP-8, and 20.8 and 56.1 ng/ml for postoperative MMP-8.

When the whole patient cohort was studied, both low and high preoperative values of MMP-8 (≤29.6 and >76.2 ng/ml) associated with shorter OS (*P*=0.023) compared to intermediate values and showed tendency towards shorter DFS (*P*=0.087). The same difference was observed more strongly among male patients (OS: *P*=0.026) and in synchronous disease (OS: *P*=0.051). Preoperatively high MMP-8 (>76.2 ng/ml) associated with shorter survival among >65-year-old patients (OS: *P*=0.005), but not among younger ones, and with shorter DFS and OS in colon cancer (DFS: *P*=0.020; OS: *P*=0.002), but not in rectal cancer. Postoperatively low values of MMP-8 (≤20.8 ng/ml) associated with better prognosis compared to higher values among >65-year-old patients (OS: *P*=0.045), in colon cancer (OS: *P*=0.020), and in synchronous disease (DFS: *P*=0.020; OS: *P*=0.002), but not in the whole cohort.

The pre- and postoperative serum concentrations of MMP-9 did not associate with survival in the whole cohort, but postoperatively elevated values (>77.7 ng/ml) associated with worse survival among >65-year-old patients (DFS: *P*=0.003; OS: *P*<0.001) (**Figure 4**).

Preoperatively elevated MPO (>218.6 ng/ml) associated with improved DFS (*P*<0.001) and OS (*P*=0.014) in the whole cohort (**Figure 5**), and the association was most significant in the following subgroups: women (DFS: *P*=0.001 and OS: *P*=0.013); ≤65-year-old patients (DFS: *P*<0.001; OS: *P*=0.004); and synchronous disease (DFS: *P*=0.001; OS: *P*=0.005) (**Table 5**). Concerning postoperatively elevated MPO (>218.6 ng/ml), no statistically significant associations were found.

In addition, we studied the prognostic value of the changes in serum concentrations between pre- and postoperative measurements in the whole patient cohort. Neither postoperative increase nor decrease associated with survival. We also combined the tissue and serum expressions of MMP-8 and MMP-9 but did not find statistically significant associations with DFS or OS. However, it is worth mentioning that all patients with preoperatively elevated serum MPO seemed to have improved prognosis independently of the tumoral expression of MMP-2, MMP-8 or MMP-9 (**Supplementary Table 1**).

Associations with neoadjuvant chemotherapy

In addition to the previously presented results, we estimated the prognostic significance of the biomarkers in primary colorectal tumors and preoperative serum in patients who received neoadjuvant chemotherapy before liver resection and in those who did not.

In patients that did not receive neoadjuvant chemotherapy before liver resection (n=40; 36.0%), low MMP-8 expression in primary tumors associated with significantly worse survival after liver resection compared to high expression (DFS: *P*=0.005 and OS: *P*=0.097). However, in patients that did receive neoadjuvant chemotherapy (n=66; 59.5%), no difference between the patients with either low and those with high MMP-8 expression was found. The same was noted for MMP-9, as in patients not having received neoadjuvant therapy, low expression in primary tumors associated with worse DFS and OS (*P*=0.006 and *P*=0.062, respectively), but in those who did receive neoadjuvant therapy, no difference between the low and high expression groups was found.

In patients that received neoadjuvant chemotherapy, low preoperative serum levels of MPO associated with significantly worse prognosis (DFS: *P*<0.001 and OS: *P*=0.020), while the difference between the low and high

concentration groups was non-significant in patients that did not receive neoadjuvant treatment (DFS: *P*=0.120; OS: *P*=0.266). Patients with low and high preoperative MMP-8 in serum also had worse survival (DFS: *P*=0.026; OS: *P*=0.043) compared to patients with intermediate values, while no difference was found in those that did not receive neoadjuvant treatment.

Discussion

In this retrospective study we demonstrated that high expression of MMP-9 in primary colorectal tumors and preoperatively elevated MPO in serum indicate improved prognosis in patients undergoing resection for colorectal liver metastases. In addition, the serum concentrations of MMP-8 and MMP-9 had some prognostic significance, especially in >65-year-old patients, in colon cancer, and in CRC with synchronous liver metastases.

Earlier, Koskensalo et al. found that positive MMP-9 expression in colorectal tumor tissue indicated better prognosis in Dukes' B disease [21], but in their study, the expression of MMP-2 or MMP-8 did not associate with prognosis. The results of our study are similar, revealing that high MMP-9 expression in primary tumor tissue associates with better DFS and OS also in stage IV disease. It can be speculated that in case the expression of MMP-9 is high in the tumor cells, MMP-9 cannot be released into the surrounding extracellular matrix and thus, the invasive capability of the tumor is eventually weaker and prognosis better.

On the other hand, the expression of MMP-9 has also been associated with poor survival [19, 20] in primary CRC, which is contradictory to our results. In our study, high MMP-9 expression associated with better prognosis specifically in patients with metachronous liver metastases, that is, stage I–III disease at the time of the primary operation. Noteworthy, MMP-9 expression in primary tumors was lower in patients with synchronous liver metastases compared to metachronous ones, which corresponds to the results of a previous study by Takeha et al. [51]. They found that the number of MMP-9-positive cells along colorectal tumors' invasive margin was significantly smaller in patients with liver metastases or with an infiltrating growth pattern. In our material, also the tissue expression of MMP-8 in primary tumors and liver metastases was lower in synchronous than in metachronous disease, even though the prognostic significance of MMP-8 was weak. It can be hypothesized that the tumoral expression pattern changes when CRC becomes metastatic, and low expression of MMP-9 in primary tumors reflects either already existing or shortly developing liver metastases and thus, worse prognosis. The reason for the difference between the prognostic

value of tissue MMP-9 in previous studies and ours remains unclear, but the association with improved prognosis has now been demonstrated in at least two studies with similar methods.

In this study, the expression of MMP-2 and MMP-8 in primary tumors did not associate with survival. MMP-2 has earlier been found prognostic in CRC [13], but it is possible that the significance changes in a metastatic setting. Concerning MMP-8, our results did not offer new information in relation to the previous findings.

We expected that the expression of MMP-2, MMP-8, or MMP-9 in liver metastases would have associated with prognosis, but only weak associations were found in some subgroups. It can be speculated that preoperative chemotherapy either affects the prognostic significance of MMPs in liver metastases or reflects the response to oncological treatment, and it may consequently alter the expression of MMPs and their associations with prognosis. In our patient cohort, a significant number of patients, 59.5%, received neoadjuvant chemotherapy before liver resection.

In this respect it is worth mentioning that all the patients in this study were operated before the year 2008, and since then, the chemotherapy options have developed considerably. In particular, the use of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFR) inhibitors has become common. VEGF promotes angiogenesis and, hence, has an important role in tumor invasion. MMP-9 in known to mobilize VEGF in ECM, and VEGF has been suggested to be a downstream effector and possibly a substrate of MMP-9 [11]. Considering this, it is possible that the prognostic significance of MMPs depends on the type of chemotherapy used, and this aspect should be taken into consideration in the following studies.

Interestingly, the tissue expressions of MMP-2, MMP-8, and MMP-9 in primary colorectal tumors and liver metastases did not correlate with each other. In addition, only MMP-8 expression in liver metastases correlated with pre- and postoperative serum MMP-8 and with preoperative serum MPO. In synchronous disease, MMP-9 expression in primary tumors correlated negatively with postoperative MMP-8 in serum. It seems that the prognostic value of MMP-2, MMP-8, and MMP-9 varies between the primary tumors and their liver metastases, and only the serum levels of MMP-8 seem to be related to the tissue expression of MMP-8 and possibly to that of MMP-9. These findings suggest that serum MMP-8, MMP-9, and MPO may be mainly produced by extratumoral tissues.

In serum, both elevated as well as elevated and low values of MMP-8 have been associated with worse prognosis in primary colorectal and gastric cancer [28, 32]. We obtained similar results, as both the high and low values before liver resection associated with shorter survival. This refers to a dual role of MMP-8 in cancer. However, the prognostic significance of serum MMP-8 in metastatic CRC should be further investigated in a larger patient cohort to confirm this.

The association of preoperatively elevated MPO in serum and improved survival after liver resection is in line with the previous results obtained in breast cancer [45]. On the other hand, low preoperative MPO concentrations indicated significantly impaired prognosis. To the best of our knowledge, there are no previously published studies on serum MPO in metastatic CRC. Our results suggest that MPO has protective characteristics in cancer, and it is possible that high concentrations signal a stronger immune response against cancer metastasis and thus, a better prognosis. This hypothesis is also supported by our finding that MPO was prognostic especially in patients with synchronous liver metastases. MPO is known to oxidatively activate proMMP-8 and -9 as well as to inactive TIMP-1, and it can be assumed that combining these biomarkers would further help estimate the risk of recurrence after liver resection, also in patients with metachronous metastases. Unfortunately, we were able to perform only analyses with limited statistical power concerning the combined prognostic value of tissue MMP-8 and MMP-9 and serum MPO because of the size of the patient cohort.

In addition, we observed that the prognostic significance of the investigated biomarkers varied according to the patients' clinical characteristics. MMP-8 in liver metastases and serum, MMP-9 in primary tumors, and preoperative MPO in serum associated with prognosis especially in colon cancer, although weaker associations in rectal cancer were also found. Pre- and postoperative serum MMP-8 as well as postoperative serum MMP-9 associated with survival more strongly in >65-year-old patients than in younger ones. Preoperative serum MPO, on the other hand, was prognostic especially in ≤65-year-old patients. This may relate to the fact that the inflammatory responses change with ageing [52], and the prognostic significance of these biomarkers alters correspondingly. It is possible that the cancer-promoting effects of MMP-8 and

MMP-9 are more efficiently compensated in younger patients. Our results suggest that age should be taken into consideration when adjusting these biomarkers to clinical use.

It is also worth mentioning that the prognostic significance of MMP-9 expression in primary tumors and that of preoperative serum MPO were stronger in women than in men, possibly referring to a connection with hormonal regulation. Stimulation of estrogen receptor α (ERα) has been shown to mediate the upregulation of MMP-9 [53] and also the activation of the *RAS*/ERK pathway, which promotes cancer progression [54]. Estrogen itself has been suggested to promote tumorigenesis in mouse models [55], and in a study by Pósa et al. [56], estrogen deficiency was associated with increased activity of MPO. Our findings refer to a possible link between estrogen, MMP-9, and MPO. High levels of MPO may associate with lower estrogen levels in some women and thus, a better prognosis. On the other hand, high estrogen levels followed by stimulation of estrogen receptors might cause the release of MMP-9 from inside the tumor cells into the extracellular matrix – observed as a low expression in the tumor cells – and, consequently, impaired prognosis.

The results concerning the differences in the biomarkers' prognostic value in patients that received or did not receive neoadjuvant chemotherapy are interesting. They suggest that the patients with low expression of MMP-8 and/or MMP-9 in primary colorectal tumors may benefit from neoadjuvant chemotherapy before liver resection, while those with high expression may not necessarily need it. Preoperative serum values of MPO and possibly MMP-8, on the other hand, may reflect the response to neoadjuvant therapy and thus, risk of recurrence and death. These results should, however, be considered with precaution because of the quite small number of patients in the subgroups.

A limitation of this study is the size of the patient cohort, because of which we were not able to evaluate the biomarkers' prognostic significance in all possible subgroups, and the analyses concerning their combined prognostic value were mostly cursory. On the other hand, the statistically significant findings most probably are reliable, since they were obvious despite of the quite small cohort. The patients' *RAS* and *BRAF* mutation status or the microsatellite instability status of the tumors would have given important additional information, but unfortunately, these were not routinely analyzed in our clinic prior to years 2013 and 2018, respectively.

The strengths of our study are the long follow-up period as well as the comprehensive and reliable follow-up data. The clinical material is unique, as it consists of tumor tissue samples of both primary CRC tumors and their liver metastases in addition to serum samples taken before and after liver resection. In the future, it would be valuable to evaluate also the expression of MPO and estrogen receptors in tumor tissue samples as well as the serum levels of TIMP-1 and estrogen in a larger cohort of patients with metastatic CRC.

In conclusion, high expression of MMP-9 in colorectal tumor tissue and preoperatively elevated MPO in serum associated with improved prognosis in patients undergoing liver resection for colorectal metastases, and low expression and low serum concentrations with poor survival. These biomarkers might identify the patients at a high risk of recurrence after liver resection and help adjust their treatment accordingly. Gender, age, and the synchronicity of the liver metastases seemed to affect the prognostic significance of these biomarkers, possibly via immunological and hormonal mechanisms, which should be investigated further. In addition, tissue expression of MMP-8 and MMP-9 in primary colorectal tumors may identify the patients that benefit from neoadjuvant chemotherapy before liver resection, and preoperative serum MPO and MMP-8 may possibly be used for monitoring response to neoadjuvant treatment.

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Statement of Ethics

This study was conducted in accordance with the Declaration of Helsinki, and it was approved by the Ethics Committee at the Helsinki University Hospital (IRB99/07/01, HUS531/E6/01, HUS460/E6/05, HUS323/13/3/2008, HUS242/13/03/02/2011, and HUS 226/E6/06, extension TMK02 §66 17.4.2013). A verbal informed consent was obtained from all the patients included in the study by the treating physician(s). At the time of the collection of the blood samples, verbal consent was generally accepted, and the Ethics Committee at the Helsinki University Hospital approved the verbal consent procedure. The permission to use the blood samples in recent studies was given and the collection and analysis of the tissue samples was approved by the National Supervisory Authority for Welfare and Health (Valvira) of Finland (STM Dno 4858/04/047/08 and Valvira Dnro 10041/06.01.03.01/2012). Information about the dates of death was obtained from the Central Statistical Office of Finland (TK-53-1004-9).

Conflict of Interest Statement

RP, JH, TT, CH, and HI declare no conflicts of interest. TS is an inventor of the U.S. patents 2017/0023572 A1, WO 2018/060553 A1, 15,121801, FI-127424, and the Japanese patent 2016-55476.

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Author Contributions

All the authors participated in interpreting results and reviewing and editing the manuscript. RP collected the frozen serum samples and selected the tissue samples from the archives, collected the clinical data, analyzed the immunohistochemically stained tissue samples, performed and interpreted all the statistical analyses, and prepared and edited the manuscript. JH analyzed the immunohistochemically stained tissue samples and took the pictures of the stained tissues. TT performed the measurements of MMP-8, MMP-9, and MPO in the serum samples. HI designed the study concept and carried responsibility for quality control of the data and the results. HI, CH, and TS determined the study concepts and enabled data acquisition. All the authors have read and agreed to the current version of the manuscript.

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Table 1. Patient characteristics.

Characteristic	**n (%) or median (minimum–maximum)**
Gender	
Male	64 (57.7)
Female	47 (42.3)
Age	
At the operation on the primary tumor	62.8 years (35.5–80.4)
At the liver resection	64.4 years (36.3–81.5)
>65 years at the primary operation	45 (40.5)
>65 years at the liver resection	53 (47.7)
Primary tumor	
Rectum	48 (43.2)
Left colon or rectosigmoid junction	44 (39.6)
Right or transversal colon	19 (17.1)
Primary TNM staging	
T1–2	16 (14.4)
T3–4	87 (78.4)
Missing data	8 (7.2)
N0	35 (31.5)
N1	41 (36.9)
N2	28 (25.2)
Missing data	7 (6.3)
Liver metastases	
Synchronous[1]	66 (59.5)
Metachronous	45 (40.5)
≤2 liver metastases	87 (78.4)
Diameter of the largest metastasis ≤2.0 cm	47 (42.3)
Resection margins	
R0 (margin histologically free)	102 (91.9)
R1 (histologic neoplastic infiltration)	8 (7.2)
Missing data	1 (0.9)
Follow-up time for patients alive	15.2 years (11.5–20.3)
Overall survival for all patients	5.7 years (0.2–20.3)
5-year disease-free survival	33 (29.7)
5-year overall survival	66 (59.5)
10-year overall survival	41 (36.9)
Recurrence after liver resection	
Yes	80 (72.1)
No	31 (27.9)
Site of recurrence	
Liver	44/80 (55.0)
Other	32/76 (40.0)
Not known	4/80 (5.0)
Neoadjuvant chemotherapy before liver resection	
Yes	66 (59.5)
No	40 (36.0)
Missing data	5 (4.5)
Adjuvant chemotherapy after liver resection	
Yes	83 (74.8)
No	17 (15.3)
Missing data	11 (9.9)

[1] Synchronous: liver metastases diagnosed within 6 months after the operation on the primary tumor.

Table 2. Tissue expressions of MMP-2, MMP-8, and MMP-9 and serum concentrations of MMP-8, MMP-9, and MPO.

Tissue biomarkers	**Colorectal tumors (N=81)** **n (%)**	**Liver metastases (N=88)** **n (%)**
MMP-2		
0	1 (1.2)	7 (8.0)
1	9 (11.1)	41 (46.6)
2	49 (60.5)	30 (34.1)
3	22 (27.2)	10 (11.4)
MMP-8		
0	6 (7.4)	8 (9.1)
1	32 (39.5)	47 (53.4)
2	36 (44.4)	31 (35.2)
3	7 (8.6)	2 (2.3)
MMP-9		
0	23 (28.4)	26 (29.5)
1	28 (34.6)	42 (47.7)
2	26 (32.1)	18 (20.5)
3	4 (4.9)	2 (2.3)
Serum biomarkers	**Preoperative concentrations (N=111), median (IQR)**	**Postoperative concentrations (N=111), median (IQR)**
MMP-8 (ng/ml)	48.3 (22.9–96.5)	33.2 (16.8–86.8)
MMP-9 (ng/ml)	79.6 (50.8–111.0)	75.9 (39.8–114.5)
MPO (ng/ml)	196.7 (132.7–341.1)	186.3 (103.4–286.0)

Table 3. Disease-free survival and overall survival in different subgroups of patients. *P*-values from log-rank test in relation to overall DFS and OS.

Clinical variable	**N=111 n (%)**	**Disease-free survival**		**Overall survival**	
		5-year DFS n (%)	***P*-value**	**10-year OS n (%)**	***P*-value**
Survival in the whole cohort	111 (100.0)	33 (29.7)		41 (36.9)	
Gender					
Male	64 (57.7)	18 (28.1)	0.974	23 (35.9)	0.883
Female	47 (42.3)	15 (31.9)		18 (38.3)	
Age at the primary operation					
≤65 years	66 (59.5)	24 (36.4)	0.036	30 (45.5)	0.013
>65 years	45 (40.5)	9 (20.0)		11 (24.4)	
Age at the liver resection					
≤65 years	58 (52.3)	22 (37.9)	0.014	26 (44.8)	0.041
>65 years	53 (47.7)	11 (20.8)		15 (28.3)	
Location of the primary tumor					
Rectum	48 (43.2)	8 (16.7)	0.126	16 (33.3)	0.362
Left colon or rectosigmoid	44 (39.6)	16 (36.4)		16 (36.4)	
Right or transversal colon	19 (17.1)	9 (47.4)		9 (47.4)	
Colon, left and right combined (vs. rectum)	63 (56.8)	25 (39.7)	0.052	25 (39.7)	0.180
Primary TNM stage					
T1–2	16 (14.4)	9 (56.3)	0.015	12 (75.0)	0.006
T3–4	87 (78.4)	23 (26.4)		28 (32.2)	
Missing data	8 (7.2)				
N0	35 (31.5)	15 (42.9)	0.111	19 (54.3)	0.011
N1–2	69 (62.2)	17 (24.6)		21 (30.4)	
Missing data	7 (6.3)				
Liver metastases					
Synchronous	66 (59.5)	17 (25.8)	0.088	22 (33.3)	0.226
Metachronous	45 (40.5)	16 (35.6)		19 (42.2)	
≤2 metastases	87 (78.4)	29 (33.3)	0.053	34 (39.1)	0.281
>2 metastases	24 (21.6)	4 (16.7)		7 (29.2)	
Largest diameter ≤2.0 cm	47 (42.3)	18 (38.3)	0.317	19 (40.4)	0.164
Largest diameter >2.0 cm	64 (57.7)	15 (23.4)		22 (34.4)	
Any neoadjuvant chemotherapy before liver resection					
Yes	66 (59.5)	21 (31.8)	0.380	24 (36.4)	0.486
No	40 (36.0)	12 (30.0)		17 (42.5)	
Missing data	5 (4.5)				
Any adjuvant chemotherapy after liver resection					
Yes	83 (74.8%)	26 (31.3)	0.447	34 (41.0)	0.159
No	17 (15.3%)	5 (29.4)		5 (29.4)	
Missing data	11 (9.9%)				

Table 4. Results of the univariate Cox regression analysis in relation to disease-free and overall survival.

Variable	Disease-free survival		Overall survival	
	HR [95% CI]	***P*-value**	**HR [95% CI]**	***P*-value**
Gender				
Male	1.00		1.00	
Female	0.99 [0.65–1.52]	0.974	1.04 [0.66–1.63]	0.883
Age at primary operation[1]	1.03 [1.01–1.05]	0.014		
Age at liver resection[1]	1.03 [1.01–1.05]	0.017	1.04 [1.01–1.06]	0.008
Primary tumor				
Rectum	1.00		1.00	
Left or right colon	0.66 [0.43–1.01]	0.054	0.74 [0.47–1.15]	0.182
Primary TNM stage				
T1–2	1.00		1.00	
T3–4	2.32 [1.16–4.65]	0.018	3.05 [1.32–7.05]	0.009
N0	1.00		1.00	
N1–2	1.46 [0.91–2.33]	0.115	1.93 [1.15–3.24]	0.013
Liver metastases				
Synchronous	1.00		1.00	
Metachronous	1.46 [0.94–2.26]	0.090	1.33 [0.84–2.12]	0.228
Number of metastases[1]	1.13 [0.98–1.29]	0.095	1.10 [0.95–1.28]	0.215
Size of the largest metastasis[1]	1.06 [0.95–1.18]	0.325	1.13 [1.00–1.26]	0.045
Liver resection				
Minor	1.00		1.00	
Major	0.87 [0.57–1.34]	0.527	0.75 [0.47–1.19]	0.223
Resection margins				
R0	1.00		1.00	
R1	1.38 [0.67–2.86]	0.387	1.37 [0.63–2.97]	0.433
Neoadjuvant chemotherapy before liver resection				
No	1.00		1.00	
Yes	1.22 [0.78–1.91]	0.382	1.19 [0.73–1.92]	0.487
Adjuvant chemotherapy				
No	1.00		1.00	
Yes	0.80 [0.45–1.43]	0.449	0.64 [0.34–1.20]	0.163
MMP-2 in tumor tissue				
Primary tumors				
Low (score 0–1)	1.00		1.00	
High (score 2–3)	1.03 [0.49–2.18]	0.931	0.79 [0.37–1.69]	0.549
Liver metastases				
Low (score 0–1)	1.00		1.00	
High (score 2–3)	0.77 [0.48–1.23]	0.286	0.75 [0.45–1.25]	0.267
MMP-8 in tumor tissue				
Primary tumors				
Low (score 0–1)	1.00		1.00	
High (score 2–3)	0.69 [0.42–1.14]	0.145	0.74 [0.43–1.28]	0.281
Liver metastases				
Low (score 0–1)	1.00		1.00	
High (score 2–3)	1.21 [0.75–1.94]	0.438	1.29 [0.77–2.14]	0.334
MMP-9 in tumor tissue				
Primary tumors				
Low (score 0–1)	1.00		1.00	
High (score 2–3)	0.50 [0.29–0.86]	0.013	0.59 [0.33–1.05]	0.070
Liver metastases				
Low (score 0–1)	1.00		1.00	
High (score 2–3)	0.99 [0.57–1.71]	0.970	0.79 [0.43–1.45]	0.442

MMP-8 in serum[2]				
Preoperative	0.95 [0.81–1.10]	0.458	1.00 [0.86–1.18]	0.967
Postoperative	1.05 [0.92–1.19]	0.479	1.11 [0.97–1.28]	0.127
MMP-9 in serum[2]				
Preoperative	0.81 [0.63–1.06]	0.121	0.94 [0.72–1.22]	0.633
Postoperative	1.03 [0.84–1.27]	0.771	1.14 [0.91–1.43]	0.255
MPO in serum[2]				
Preoperative	0.76 [0.59–0.97]	0.026	0.80 [0.62–1.02]	0.076
Postoperative	0.90 [0.75–1.09]	0.289	0.95 [0.78–1.17]	0.636

[1] Continuous variable.
[2] Log_2-transformed continuous variable.

Table 5. Tissue expression of MMP-9 in primary colorectal tumors and preoperative serum concentrations of MPO in specified subgroups of patients in relation to disease-free and overall survival. *P*-values from log-rank test in relation to overall DFS and OS. MMP-9: low: score 0–1; high: score 2–3; MPO: low: ≤218.6 ng/ml; high: >218.6 ng/ml.

Subgroup	**Disease-free survival**			**Overall survival**		
	5-year DFS, n (%)			**10-year OS, n (%)**		
MMP-9 in primary tumors	**Low**	**High**	***P*-value**	**Low**	**High**	***P*-value**
All patients (N=111)	14/51 (27.5)	15/30 (50.0)	0.010	16/51 (31.4)	16/30 (53.3)	0.067
Gender						
Male	8/28 (28.6)	6/16 (37.5)	0.422	10/28 (35.7)	7/16 (43.8)	0.841
Female	6/23 (26.1)	9/14 (64.3)	0.005	6/23 (26.1)	9/14 (64.3)	0.014
Age at liver resection						
≤65 years	11/32 (34.4)	9/15 (60.0)	0.054	12/32 (37.5)	11/15 (73.3)	0.045
>65 years	3/19 (15.8)	6/15 (40.0)	0.017	4/19 (21.1)	5/15 (33.3)	0.334
Primary tumor						
Rectum	2/13 (15.4)	4/15 (26.7)	0.076	3/13 (23.1)	6/15 (40.0)	0.360
Colon	12/38 (31.6)	11/15 (73.3)	0.011	13/38 (34.2)	10/15 (66.7)	0.037
Liver metastases						
Synchronous	11/37 (29.7)	5/13 (38.5)	0.515	12/37 (32.4)	4/13 (30.8)	0.991
Metachronous	3/14 (21.4)	10/17 (58.8)	0.005	4/14 (28.6)	12/17 (70.6)	0.032
Preoperative MPO in serum	**Low**	**High**	***P*-value**	**Low**	**High**	***P*-value**
All patients (N=111)	9/60 (15.0)	23/48 (47.9)	<0.001	15/60 (25.0)	25/48 (52.1)	0.014
Gender						
Male	6/35 (17.1)	11/27 (40.7)	0.073	9/35 (25.7)	13/27 (48.1)	0.281
Female	3/25 (12.0)	12/21 (57.1)	0.001	6/25 (24.0)	12/21 (57.1)	0.013
Age at liver resection						
≤65 years	6/32 (18.8)	16/24 (66.7)	<0.001	10/32 (31.3)	16/24 (66.7)	0.004
>65 years	3/28 (10.7)	7/24 (29.2)	0.124	5/28 (17.9)	9/24 (37.5)	0.534
Primary tumor						
Rectum	1/27 (3.7)	7/20 (35.0)	0.010	5/27 (18.5)	11/20 (55.0)	0.051
Colon	8/33 (24.2)	16/28 (57.1)	0.017	10/33 (30.3)	14/28 (50.0)	0.128
Liver metastases						
Synchronous	4/37 (10.8)	12/27 (44.4)	0.001	7/37 (18.9)	14/27 (51.9)	0.005
Metachronous	5/23 (21.7)	11/21 (52.4)	0.102	8/23 (34.8)	11/21 (52.4)	0.638

Figure 1. Study flow diagram.

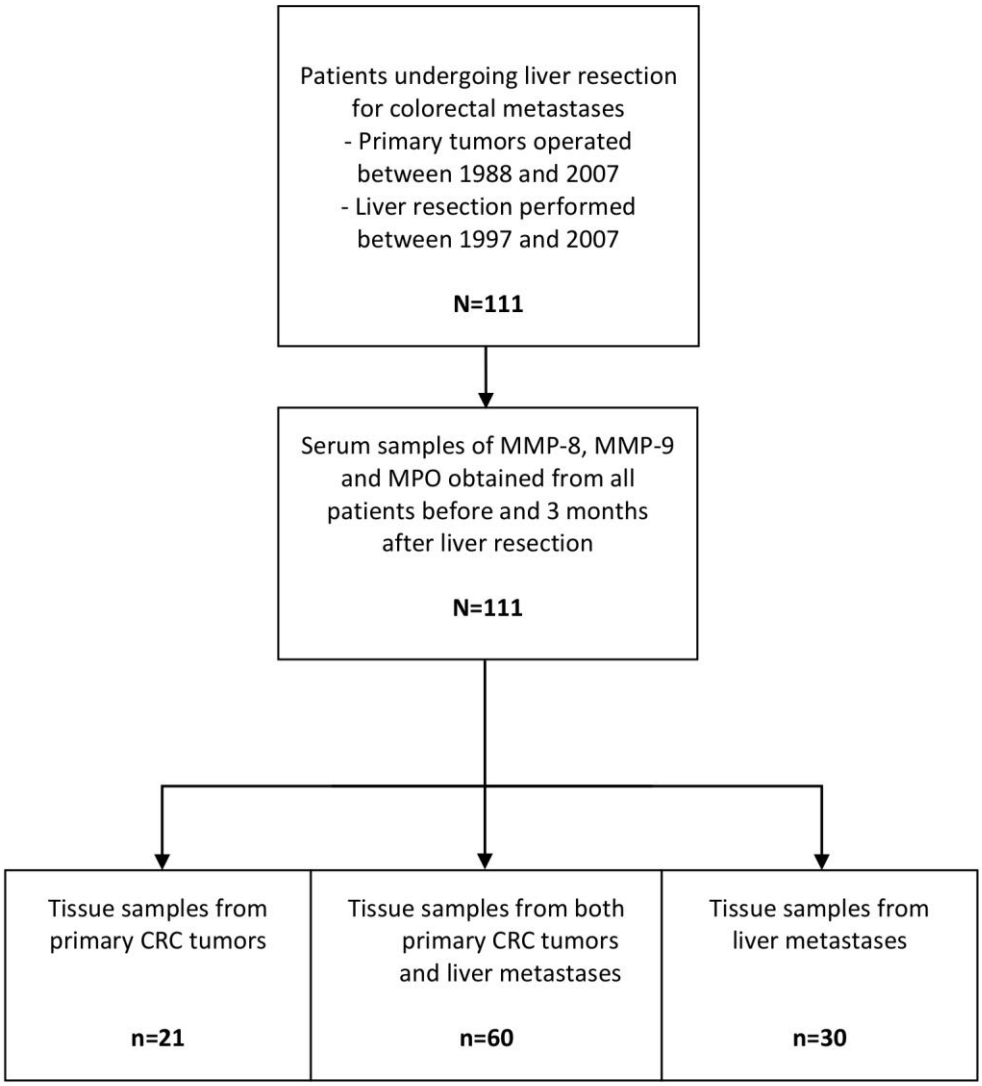


Figure 2. Immunohistochemical staining of MMP-2, MMP-8, and MMP-9 in A) primary colorectal tumors, and B) liver metastases. MMP-2: cytoplasmic staining in tumor cells; MMP-8: staining in inflammatory cells; MMP-9: granular cytoplasmic staining in tumor cells. Original magnification at x20.

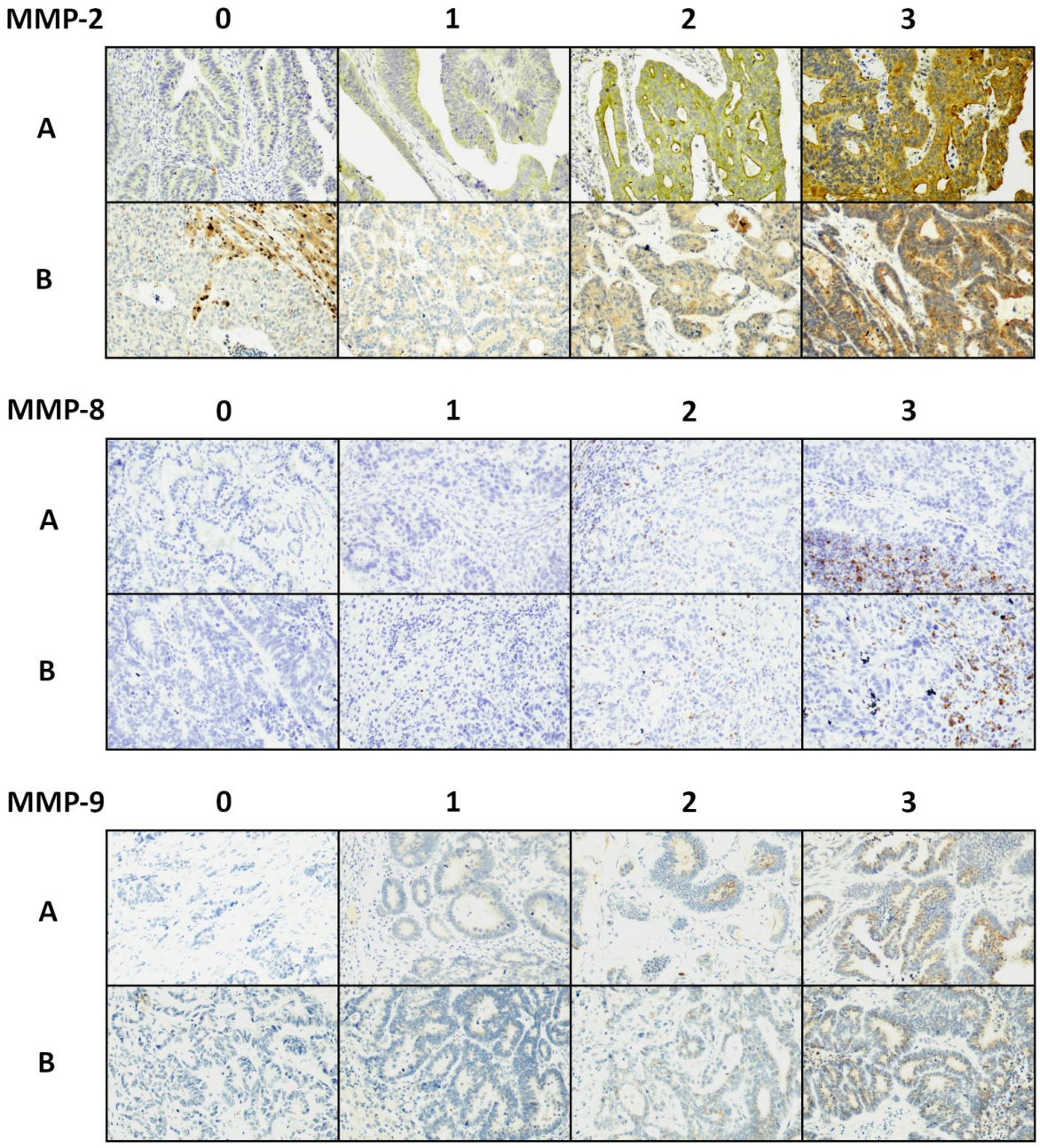
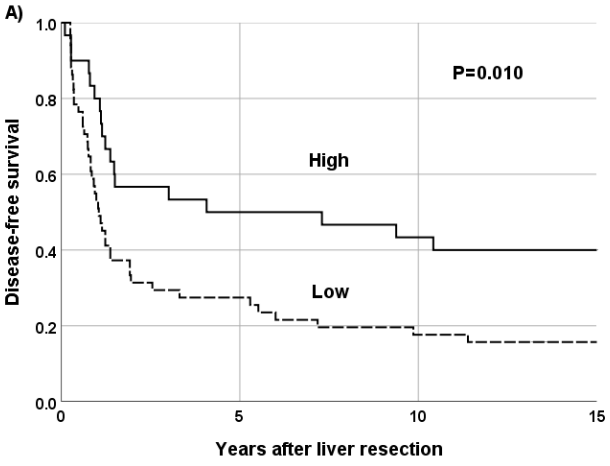
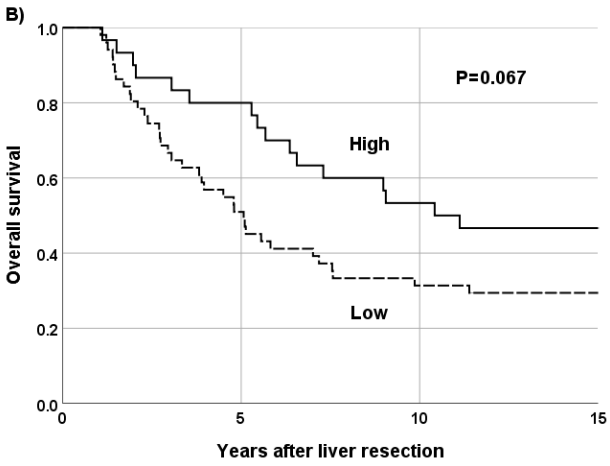


Figure 3. The expression of MMP-9 in primary colorectal tumors in relation to A) disease-free survival, and B) overall survival.



Patients at risk (n)

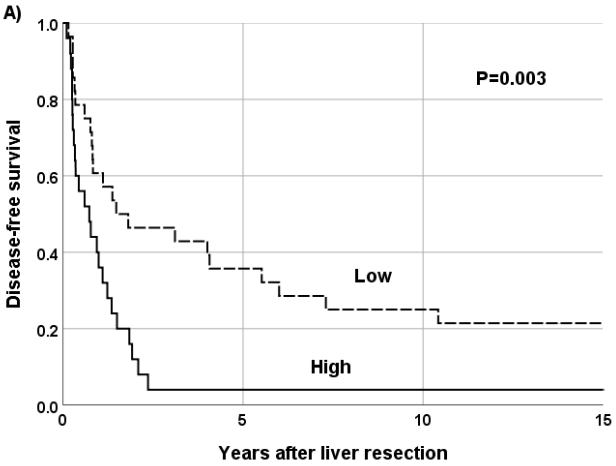
MMP-9 | years	0	3	5	10	15
Low (score 0-1)	51	15	14	9	2
High (score 2-3)	30	17	15	13	7



Patients at risk (n)

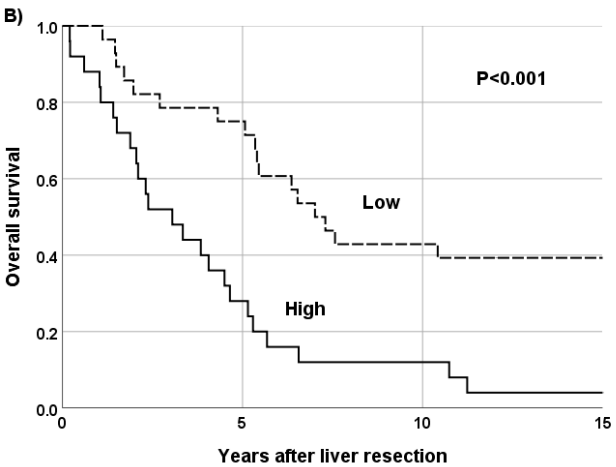
MMP-9 | years	0	3	5	10	15
Low (score 0-1)	51	34	26	16	6
High (score 2-3)	30	26	24	16	9

Figure 4. Postoperative serum concentrations of MMP-9 in >65-year-old patients in relation to A) disease-free survival, and B) overall survival.



Patients at risk (n)

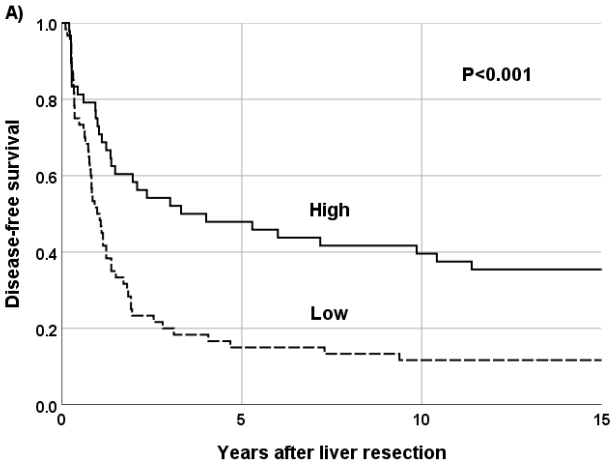
MMP-9 | years	0	3	5	10	15
Low (<=77.7 ng/ml)	28	13	10	7	1
High (>77.7 ng/ml)	25	1	1	1	1



Patients at risk (n)

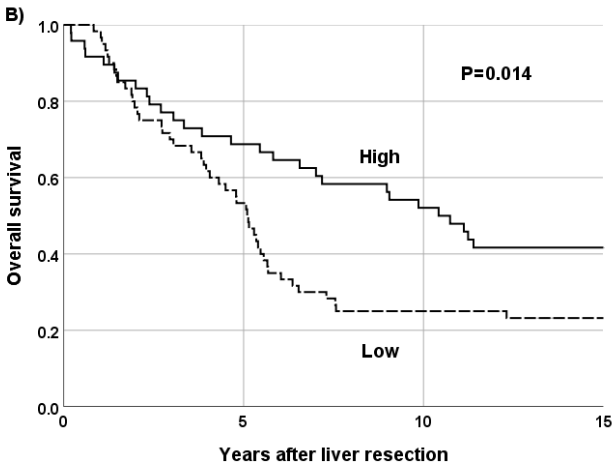
MMP-9 | years	0	3	5	10	15
Low (<=77.7 ng/ml)	28	22	21	12	5
High (>77.7 ng/ml)	25	13	7	3	1

Figure 5. Preoperative serum concentrations of MPO in relation to A) disease-free survival, and B) overall survival.



Patients at risk (n)

MPO | years	0	3	5	10	15
Low (<=218.6 ng/ml)	60	12	9	7	2
High (>218.6 ng/ml)	48	26	23	19	9



Patients at risk (n)

MPO | years	0	3	5	10	15
Low (<=218.6 ng/ml)	60	42	32	15	7
High (>218.6 ng/ml)	48	37	33	25	11

Supplementary Table 1. Preoperative serum concentrations of MPO in relation to 5-year disease-free survival and 10-year overall survival in the subgroups of patients with low/high tissue expression of MMP-2, MMP-8, and MMP-9. *P*-values (log-rank test) represent comparison of the MPO levels in the subgroups. MPO: low: ≤218.6 ng/ml; high: >218.6 ng/ml; tissue biomarkers: low: score 0–1; high: score 2–3.

	5-year disease-free survival			**10-year overall survival**		
	n (%)			**n (%)**		
MPO	**Low**	**High**	***P*-value**	**Low**	**High**	***P*-value**
Biomarkers in colorectal cancer tissue and serum MPO						
MMP-2 Low High	 1/5 (20.0) 8/41 (19.5)	 4/5 (80.0) 15/28 (53.6)	 0.104 0.002	 1/5 (20.0) 12/41 (29.3)	 2/5 (40.0) 16/28 (57.1)	 0.136 0.020
MMP-8 Low High	 2/20 (10.0) 7/26 (26.9)	 9/16 (56.3) 10/17 (58.8)	 0.009 0.030	 3/20 (15.0) 10/26 (38.5)	 8/16 (50.0) 10/17 (58.8)	 0.026 0.105
MMP-9 Low High	 4/34 (11.8) 5/12 (41.7)	 9/15 (60.0) 10/18 (55.6)	 0.002 0.442	 8/34 (23.5) 5/12 (41.7)	 7/15 (46.7) 11/18 (61.1)	 0.064 0.273
Biomarkers in liver metastasis tissue and serum MPO						
MMP-2 Low High	 3/30 (10.0) 1/18 (5.6)	 8/17 (47.1) 10/21 (47.6)	 0.012 0.001	 8/30 (26.7) 3/18 (16.7)	 8/17 (47.1) 13/21 (61.9)	 0.220 0.002
MMP-8 Low High	 5/33 (15.2) 1/17 (5.9)	 10/20 (50.0) 6/16 (37.5)	 0.003 0.112	 9/33 (27.3) 3/17 (17.6)	 12/20 (60.0) 7/16 (43.8)	 0.003 0.532
MMP-9 Low High	 3/37 (8.1) 1/11 (9.1)	 16/30 (53.3) 2/8 (25.0)	 <0.001 0.124	 6/37 (16.2) 4/11 (36.4)	 16/30 (53.3) 5/8 (62.5)	 0.001 0.385